– EXAMPLE 7

GENETIC BIT ANALYSIS

DNA Samples. Genomic DNA was isolated using the SDS/Proteinase K procedure (Maniatis, T. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) from peripheral blood nucleated cells of humans or horses enriched from red blood cells by selective lysis accomplished by diluting blood with a three fold volume excess of ACK lysing buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA). Oligonucleotides were prepared by solid-phase phosphoramidite chemistry using an Applied Biosystems, Inc. (Foster City, CA) Model 391 automated DNA synthesizer. In the case of primers used in Genetic Bit Analysis (GBA) reactions, detritylation was not performed following the final cycle of synthesis and the full-length oligonucleotide was purified using the Applied Biosystems oligonucleotide purification cartridge (OPC) as recommended by the manufacturer. For most PCR reactions, primers were used directly by drying down the de-protection reaction. Oligonucleotides derivatized with 5'-amino groups were prepared using Aminolink 2 purchased from Applied Biosystems and used according to the manufacturer's recommendations.

Template Preparation. Amplification of genomic sequences was performed using the polymerase chain reaction (PCR) (Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., Primer Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science 239:487-491). In a first step, one hundred nanograms of genomic DNA was used in a reaction mixture containing each first round primer at a concentration of 2μM/10 mM Tris pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% gelatin/0.05 units per μl Taq DNA Polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT). Reactions were assembled and incubated at 94 °C for 1.5 minutes, followed by 30 cycles of 94°C/1 minute, 60 °C/2 minutes, 72 °C/3 minutes. Single stranded DNA was prepared in a second "asymmetric" PCR in which the products of the first reaction were diluted 1/1000. One of the primers was used at the standard concentration of 2 μM

while the other was used at 0.08 μM. Under these conditions, both single stranded and double stranded molecules were synthesized during the reaction.

Solid phase immobilization of nucleic acids. GBA reactions were performed in 96-well plates (Nunc Nunclon plates, Roskilde, Denmark). The GBA primer was covalently coupled to the plate by incubating 10 pmoles of primer having a 5′ amino group per well in 50 μl of 3 mM sodium phosphate buffer, pH 6, 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) overnight at room temperature. After coupling, the plate was washed three times with 10mM Tris pH 7.5/150mM NaCl/.05% polysorbitan-20 (Tween-20) ("TNTw").

Biotinylated ddNTPs. Biotinylated ddNTPs were synthesized according to U.S. Patent No. 5,047,519.

GBA in Microwell Plates. Hybridization of single-stranded DNA to primers covalently coupled to 96-well plates was accomplished by adding an equal volume of 3M NaCl/50 mM EDTA to the second round asymmetric PCR and incubating each well with 20 μl of this mixture at 55°C for 30 minutes. The plate was subsequently washed three times with TNTw. Twenty (20) μl of polymerase extension mix containing ddNTPs (3 μM each, one of which was biotinylated/5 mM DTT/7.5 mM sodium isocitrate/5 mM $MnCl_2$/0.04 units per μl of modified T7 DNA polymerase and incubated for 5 minutes at room temperature. Following the extension reaction, the plate was washed once with TNTw. Template strands were removed by incubating wells with 50 μl 0.2N NaOH for 5 minutes at room temperature, then washing the wells with another 50 μl 0.2N NaOH. The plate was then washed three times with TNTw. Incorporation of biotinylated ddNTPs was measured by an enzyme-linked assay. Each well was incubated with 20 μl of streptavidin-conjugated horseradish peroxidase (1/1000 dilution in TNTw of product purchased from BRL, Gaithersburg, MD) with agitation for 30 minutes at room temperature. After washing 5 times with TNTw, 100 μl of o-phenylenediamine (OPD, 1 mg/ml in 0.1 M Citric acid, pH 4.5) (BRL) containing 0.012% H_2O_2 was added to each well. The amount of bound enzyme was determined by photographing the plate after stopping the reaction or quantitatively using a Molecular Devices model "Vmax" 96-well spectrophotometer. --